MOLECULAR REPRODUCTION AND DEVELOPMENT 36:424–433 (1993)

Derivation and Characterization of Putative Pluripotential Embryonic Stem Cells From Preimplantation Rabbit Embryos

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ABSTRACT We have derived putative embryonic stem (ES) cell lines from preimplantation rabbit embryos and report here their initial characterization. Two principal cell types emerged following serial passage of explanted embryos, and each has subsequently given rise to immortalized cell lines. One cell type has morphology identical to primary outgrowths of trophectoderm, is strictly feeder-cell dependent, and spontaneously forms trophectodermal vesicles at high cell density. The second type appears to represent pluripotent ES cells derived from the inner cell mass as evidenced by 1) ability to grow in an undifferentiated state on feeder layers, 2) maintenance of a predominantly normal karyotype through serial passage (over 1 year), and 3) ability to form embryoid bodies, which form terminally differentiated cell types representative of ectoderm, mesoderm, and endoderm. These ES cells may ultimately be suitable for introduction of germline mutations (via homologous recombination). The rabbit's size, reproductive capability, and well-characterized physiology make it suitable for a wide range of investigations, particularly for development of large animal models of human disease.

Key Words: ES cells, Trophectoderm, Inner cell mass

INTRODUCTION

The establishment in culture of pluripotent embryonic stem (ES) cells from the mouse has ushered in an exciting new era in the study of mouse molecular genetics (Capecchi, 1989). These cells, originally derived from the proliferating inner cell mass of preimplantation embryos (Evans and Kaufman, 1981; Martin, 1981), can be passaged indefinitely in an undifferentiated state. When reintroduced into mouse blastocysts, they are capable of contributing to all tissues of the resulting (chimeric) mice. Most importantly, they contribute to the cells that eventually become germ cells, thus giving rise to germline chimeras. These properties allow for the production of new mutations, either by random integration of proviruses (Conlon et al., 1991), of "gene-trap" vectors (Gossler et al., 1989; Friedrich and Soriano, 1991), or via homologous recombination with the resident gene (for recent review, see Pascoe et al., 1992). Of these, the latter permits precise design and study of mutations in an animal.

While the mouse has proved to be extremely useful in this endeavor, and undoubtedly will remain the preferred animal for mammalian developmental biology, its size limits its usefulness for many potential applications of transgenic technology. For example, the introduction of precise mutations by homologous recombination has major potential for the development of animal models of genetic and acquired human diseases that can be used to test novel therapeutic strategies. Although it is neither practical nor feasible to perform certain types of manipulations in mice, such as invasive hemodynamic measurements or detailed metabolic studies that require frequent blood sampling, these analyses are performed without difficulty in larger animals. The advantages of larger animals are particularly apparent for studies of vascular disease, cardiomyopathy, disorders of the central nervous system, abnormalities of lipoprotein metabolism, and pulmonary physiology, where survival surgery following therapeutic interventions may be critical. Furthermore, targeted disruption of mouse homologues of certain genes that cause human disease, e.g., hypoxanthine phosphoribosyltransferase (Kuehn et al., 1987) and CFTR (Snouwaert et al., 1992), have not faithfully reproduced the desired or expected clinical phenotype.

The potential mammals in which ES cell techniques might be routinely applied are limited and, in practical terms, depend principally on gestation period and age to sexual maturity. Obviously other factors must be considered when selecting an animal species for these studies, such as ease in housing, manipulation of embryos, litter size, ability to superovulate in order to obtain adequate numbers of embryos for derivation of cell lines and blastocysts for injection, and cost to maintain. Furthermore, the animal should be a candidate for a host of investigative areas ranging from developmental biology, physiology, surgery, and metabolism, and preferably is one that investigators already utilize in the laboratory. The rabbit satisfies most of these criteria; its size and physiology have made it a favorite for

Received March 17, 1993; accepted May 4, 1993.

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numerous types of investigations for decades. It has a relatively short gestation period of 31 days (compared to 19 days for the mouse) and generally reaches sexual maturity by 5–7 months of age. In addition, the females are induced ovulators (the act of coitus induces ovulation) and conditions for superovulation have been well defined (Kennelly and Foote, 1965).

Putative ES cells have been derived from hamster (Doetschman et al., 1988), pig (Notarianni et al., 1990), cow (Saito et al., 1992), and mink (Sukoyan et al., 1992) preimplantation embryos; however, the mouse is the only species in which proven, totipotent ES cells have been derived and is currently the only mammal for which strategies for genetic manipulation of endogenous genes have been achieved. This report describes the derivation and initial characterization of putative ES cells from preimplantation rabbit embryos and the potential applications of this technology. We envisage that this endeavor will be most useful in the creation of rabbit models of diseases for which novel approaches to therapy would be facilitated by use of a large mammal.

MATERIALS AND METHODS

Reagents

Murine leukemia inhibitory factor (LIF) and DMEM were obtained from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Antidesmin antibodies were obtained from Sigma (St. Louis, MO) and used according to the manufacturer's protocols.

Animals

Adult Dutch-belted (DB) and New Zealand white (NZW) rabbits were obtained from commercial sources (Myrtle's Rabbits, Knoxville, TN). This particular colony of DB rabbits has been maintained by parent-sib matings (estimated ten generations) since Caesarian-derivation of litters 7 years prior to initiation of this study. DB does were mated with two fertile bucks of the same breed in order to obtain embryos. Mice (either FVB/N or ICR strain), for preparation of mouse embryonic fibroblasts, were obtained from Harlan-Sprague Dawley (Indianapolis, IN).

Cell Culture

Rabbit blastocysts were flushed from the uteri of naturally mated does on day 4 or day 5 postcoitus (pc) and rinsed in phosphate-buffered saline (PBS), and the mucin coat/zona pellucida was removed either mechanically (using sterile needles) or enzymatically with Pronase (0.5% in PBS). The day 4 and day 5 blastocysts were expanded blastocysts with a clearly visible inner cell mass and blastocoel cavity. These were immediately placed individually onto mitomycin C-arrested mouse embryonic fibroblasts (MEFs) or SNL76/6 feeders in 96 well culture dishes. Explanted embryos generally attached within 48 h and were then fed every other day with fresh ES media [consisting of high-glucose DMEM supplemented with 15% FBS, 0.5% nonessential amino acids, penicillin and streptomycin, 0.1 mM 2-mercaptoethanol, 4 mM glutamine and 1,000 U murine leukemia inhibitory factor (LIF)/ml]. On day 7 following placement in culture, the presumptive inner cell mass was mechanically disrupted as described elsewhere (Robertson, 1987), or the entire well was lightly trypsinized and replated onto 96 well dishes. ES-like colonies were subsequently passaged onto larger culture dishes at ~5-day passage intervals, at which time individual colonies were subcloned using sterile cloning cylinders. Trypsinized colonies were then placed back into individual 96 well dishes until sufficient cell density allowed eventual expansion onto 10 cm plates (generally by passage 7) for subsequent experiments.

MEFs and SNL76/6 feeders (kindly provided by Allan Bradley) were plated at 2–3 million cells per 10 cm dish (determined empirically to provide a confluent layer of feeder cells). Media for embryoid body formation consisted of DMEM supplemented with 10% FBS, 4 mM glutamine, and penicillin/streptomycin. ES cells were routinely passaged at subconfluence (less than ten colonies per 100× field) onto fresh, mitotically arrested SNL76/6 feeders or MEFs and were discarded if evidence of substantial differentiation or contamination with trophectodermal outgrowth was evident.

Electron Microscopy and Antibody Staining

Tissue culture dishes containing embryoid bodies (EBs) were fixed in 1:1 acetone:methanol at −20°C for 5 min prior to antibody staining. Differentiation of EBs was monitored daily until areas containing either myotubes or ciliated epithelium were identified by routine phase microscopy, at which time they were embedded for electron microscopy. Thin sectioning and staining were performed by standard techniques (Luft, 1961).

RESULTS

Embryo Manipulations and Derivation of ES Cell Lines

We chose the Dutch-belted rabbit strain for derivation of ES cell lines since its coat color (black and white) is dominant in the albino strains. Thus, once putative ES cells have been appropriately characterized, they can be injected into blastocysts from the albino strain(s) and a phenotype (coat or eye color) may be ascertained at birth, analogous to the situation for murine ES cells. This approach would facilitate the screening of putative ES cell lines for their ability to produce chimeric animals.

Figure 1A shows the morphology of explanted preimplantation rabbit embryos (day 5) growing on mitotically arrested mouse embryonic feeder (MEFs) cells after 7 days in culture. The proliferation of trophectodermal cells defines the outer aspect of each explanted embryo. At this time point the presumptive inner cell mass reaches a size deemed adequate for subsequent trypsinization or mechanical disruption (Robertson, 1987). If these embryos are allowed to grow in culture for 3–5 additional days, the proliferating inner cell mass cells

migrate to the outer aspect of the more slowly growing trophectodermal boundaries and begin to differentiate. We have derived two independent types of cell lines from these explanted embryos. One type displays morphology identical to primary outgrowths of trophectoderm and represented the most frequent isolate (indeed, it frequently overgrew the early ES-like colonies once trypsinized). It is capable of spontaneous vesicle formation when grown at high cell density (Fig. 1B). This cell type is strictly dependent on feeder cells and rapidly ceases cell division in their absence (but does not die). We presume that these cell lines represent trophectodermal cells, and further characterization is underway.

The second cell type is shown in Figure 1C and represents presumptive pluripotent ES cells derived from the inner cell mass. These grow as colonies (generally <100 cells) of small cells with one or two prominent nucleoli and a high nuclear:cytoplasmic ratio. They are slow growing, requiring an average 5 days between passage, and are also strictly feeder dependent; in the absence of feeder layers, the cells rapidly differentiate. Their morphology, typically described as "epithelioid," closely resembles that of pig ES cells (Notarianni, 1990). GM3, the cell line we have characterized most extensively, has been passaged in tissue culture for >1 year and retains the morphology shown. In addition, it retains a predominantly normal diploid karyotype (>80% of cells in metaphase spreads have 44 chromosomes; we have not yet determined the sex of the cell lines; data not shown) and readily forms embryoid bodies (see below). It is not yet clear whether exogenous growth factors such as LIF, a growth factor sufficient to maintain some mouse ES cells in the undifferentiated state (Williams et al., 1988), are necessary for its maintenance.

Table 1 lists the results of several different experiments undertaken to derive the cell lines. Fifteen independent putative ES cell lines, in addition to two trophectodermal cell lines, have been established to date. Several important points must be addressed at this juncture. Attempts to disaggregate the proliferating inner cell mass with capillary techniques, a procedure used for the derivation of mouse and pig ES cells, were unsuccessful in the rabbit; no established cell lines were obtained. This initial failure prompted us to explant *individual* embryos into 96 well dishes and to passage the entire outgrowth at high cell density until cloning cylinders could be used for subcloning the ES-like colonies. Although the reasons for failure to derive ES cells by capillary techniques are not entirely clear, it is most likely due to fragility of the cells, differentiation, and inherently low cloning efficiency. It is also possible the inner cell mass cells (or the trophectodermal cells) produce autocrine growth factors themselves, thus requiring a high cell density (or coculture) for successful establishment in culture. In addition, attempts to derive these cell lines on rabbit embryo fibroblasts (harvested from day 16 rabbit embryos by a technique analogous to that used for MEFs) were unsuccessful; rabbit fibroblasts appeared to promote differentiation into a spindle-shaped cell type, which did not passage (not shown). Interestingly, this property has also been observed for derivation of hamster ES cell lines (Doetschman et al., 1988). We have only recently established these cell lines on SNL76/6 feeders, which overproduce murine LIF; preliminary data indicate that there is no difference in their cloning efficiency or morphology. As shown, no differences were noted in our ability to derive cell lines from either day 4 or day 5 expanded blastocysts. Blastocysts at these developmental time points have a clearly defined blastocoel cavity and inner cell mass; the principal difference appears to be proliferation of the trophectodermal component. The estimated cloning efficiency (No. of ES cell lines established/No. of embryos recovered) of the established rabbit cell lines is similar to that of mouse ES cell lines.

Cellular Differentiation—Embryoid Body Formation

Under certain culture conditions, ES cells can be induced to aggregate, a process that results in embryoid body (EB) formation. After a period of time, these EBs begin to form terminally differentiated cell types, a process that defines their pluripotential capability in tissue culture. This is most easily accomplished by allowing the ES cells to grow to high cell density with subsequent passage onto a nonadherent surface (in the absence of MEFs). After several weeks in tissue culture, a number of terminally differentiated cell types can be identified. Figure 2 shows some of the structures present in culture following passage (approximate passage 15) of the rabbit ES cells onto bacteriologic dishes. Figure 2A illustrates a nonattached embryoid body following aggregation of rabbit ES cells (36 h after light trypsinization); when these later attach to the surface of the dish, they differentiate into multiple cell types. One of these cell types is shown in Figure 2B. This particular attached EB contains several clones of eccentrically localized cells (note the "saddle-shaped" area and a less dense area just above it) containing dark cytoplasmic inclusions of pigment that very likely represent neural crest-derived pigment cells. In addition, monolayers of cells that spontaneously form multinucleated tubes and stain positively for muscle-specific genes also form (Fig. 2C); these probably represent smooth or skeletal muscle cells (or both). The most striking feature of EBs in culture is the aggregates that spontaneously and rhythmically contract. Subsequent immunostaining of these cultures with antibodies to desmin, a muscle-specific intermediate filament protein, revealed patches of cells that stained positively (Fig. 2D). These latter cell types probably represent cardiomyocytes and are similar to EBs in the mouse (Sanchez et al., 1991). Undifferentiated rabbit ES cell lines did not express desmin (not shown).

Electron Microscopy of Embryoid Bodies

The appearance of terminally differentiated cell types following prolonged culture prompted an investi-

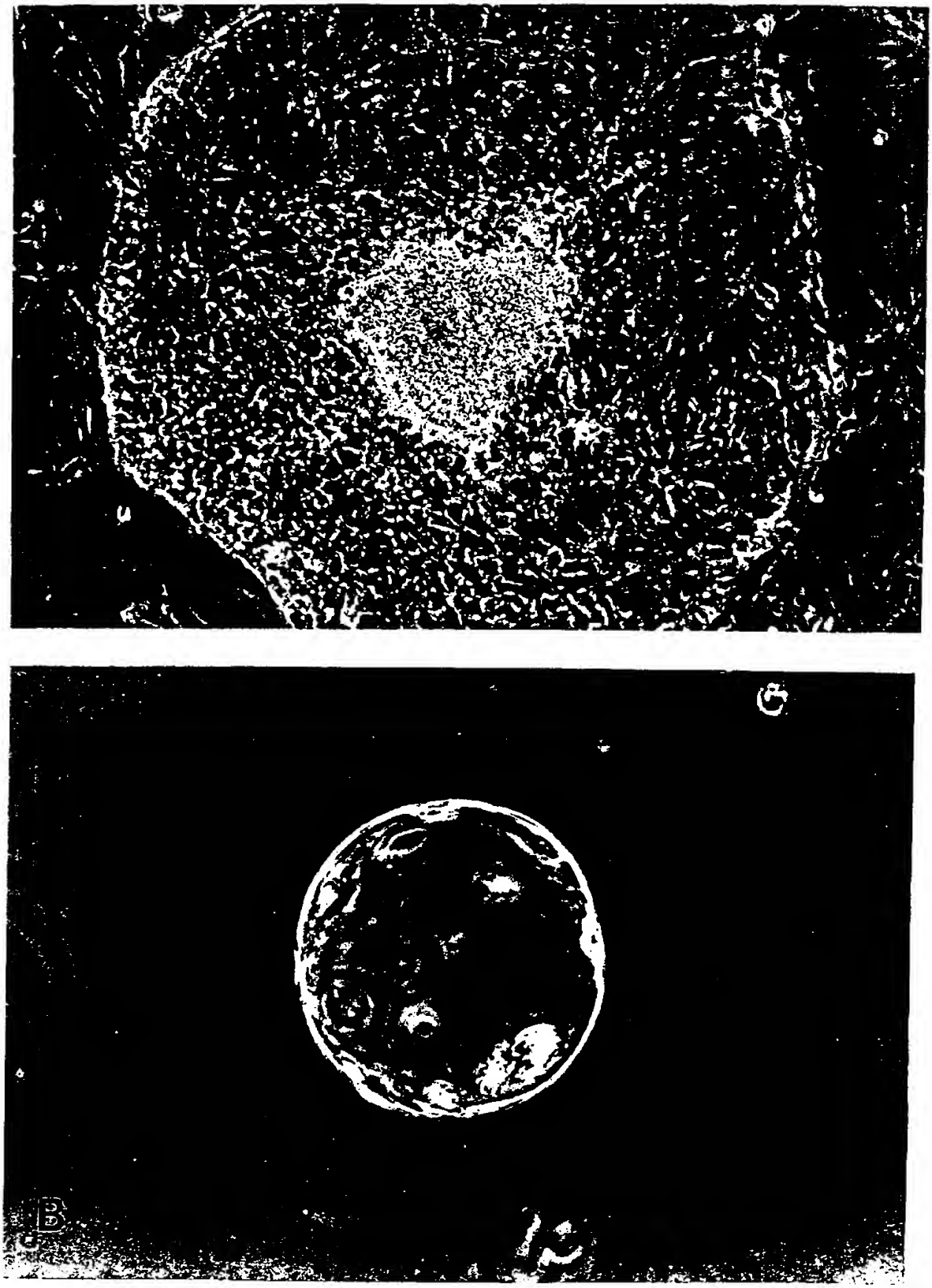


Fig. 1. A: Appearance of explanted rabbit embryo at day 7. Note the MEFs, trophectodermal outgrowth, and proliferation of the inner cell mass. B: Spontaneous vesicle formation with the trophectodermal cell line GM 1. ×40. (Figure 1 continued on next page.)

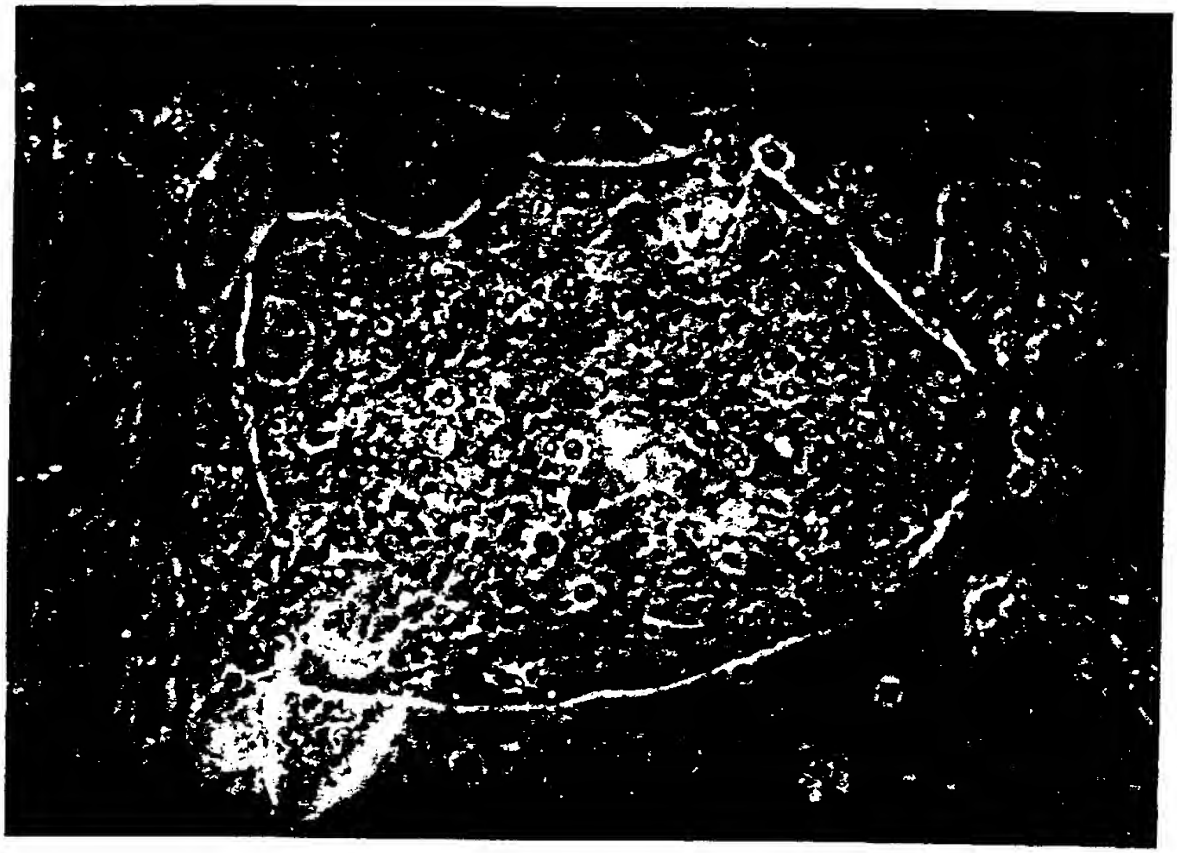


Fig. 1. C: Appearance of early passage rabbit ES cells (GM 3). ×200.

TABLE 1. Derivation of Rabbit ES Cell Lines*

Blastocyst age (days PC)	No. of embryos	No. explanted (%)	No. passaged (%)	ES lines (%)
4	80	57 (71)	36 (45)	6 (8)
5	106	80 (75)	31 (29)	9 (8)
Total	186	137 (73)	67 (36)	15 (8)

*Number explanted represents the actual number giving rise to explants as shown in Figure 1. No. passaged refers to the number subsequently giving rise to ES-like outgrowth following the initial trypsinization of the explanted embryo. Numbers in parentheses are the percentages compared to number of embryos recovered.

gation to identify additional types of cells, some of which might be quite rare. Indeed, in EBs cultured for 3–6 weeks on bacteriologic dishes, ciliated epithelium was occasionally observed. Since this appeared to be a rare event (<1% of EBs) and the characterization of these by labelling or antibody methods seemed unlikely, we chose to embed them and to perform electron microscopy instead. Cultures of EBs containing patches of ciliated epithelium or contracting myotubes (identified by phase microscopy) were embedded in acrylic, sectioned, and processed for electron microscopy. These results are shown in Figure 3. Ciliated epithelium was readily identified (Fig. 3A). In addition, the typical features of striated muscle—myofibers with a longitudinal array of actin-myosin filaments within the cytoplasm—were also easily identified (Fig. 3B). Routine passage of the undifferentiated rabbit ES cell lines has never given rise to these structures. The cell types we have observed and that are described above represent derivatives of all three germ layers—endoderm (ciliated epithelium), mesoderm (muscle), and ectoderm (pigmented epithelium)—and as such show quite conclusively that the rabbit ES cells we have derived are pluripotential.

DISCUSSION

This report describes the first successful isolation of presumptive ES cells from the rabbit as well as preliminary characterization of their in vitro properties. Two independent cell types have been derived; one appears to represent trophectoderm as evidenced by its mor-

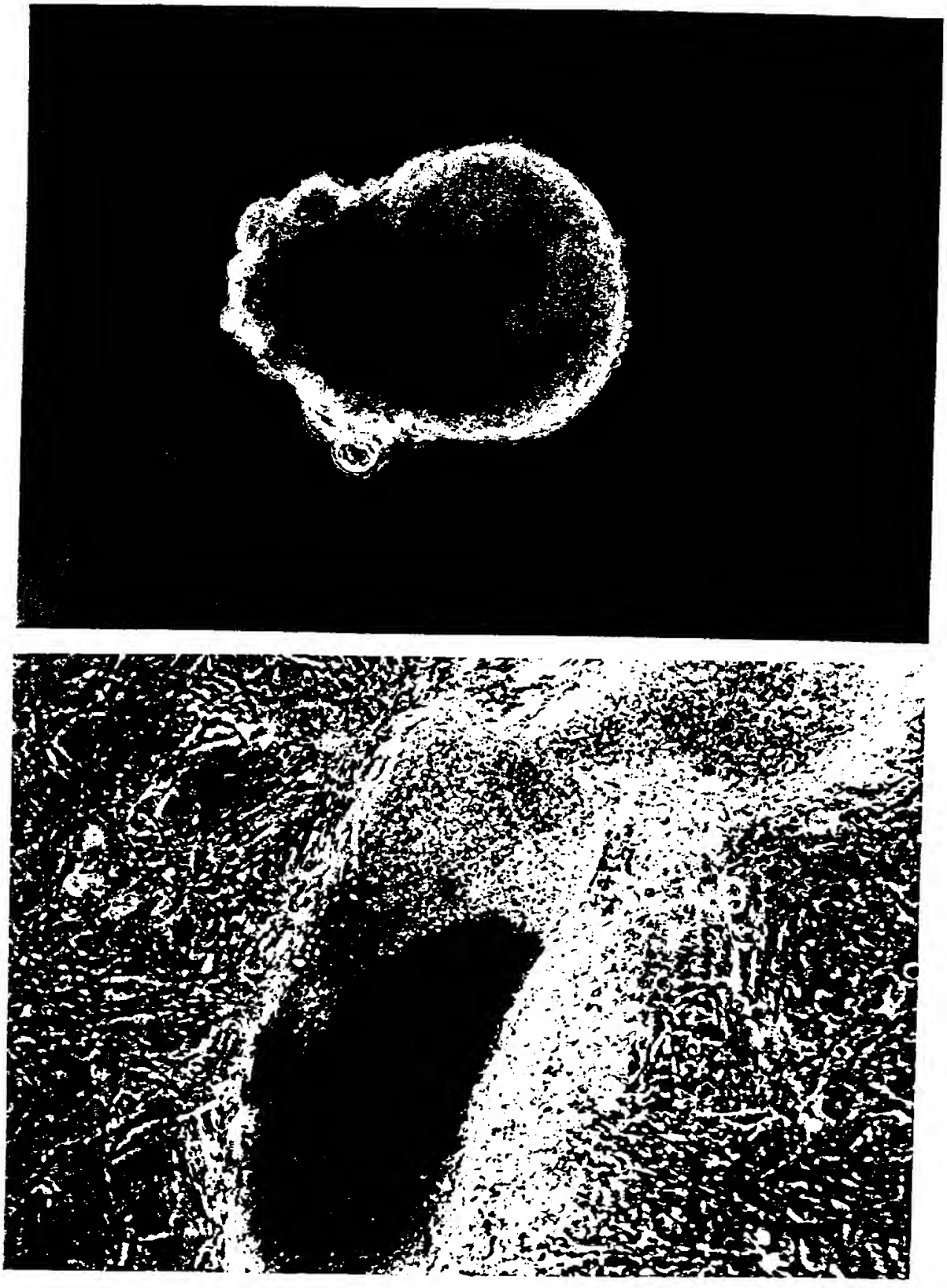
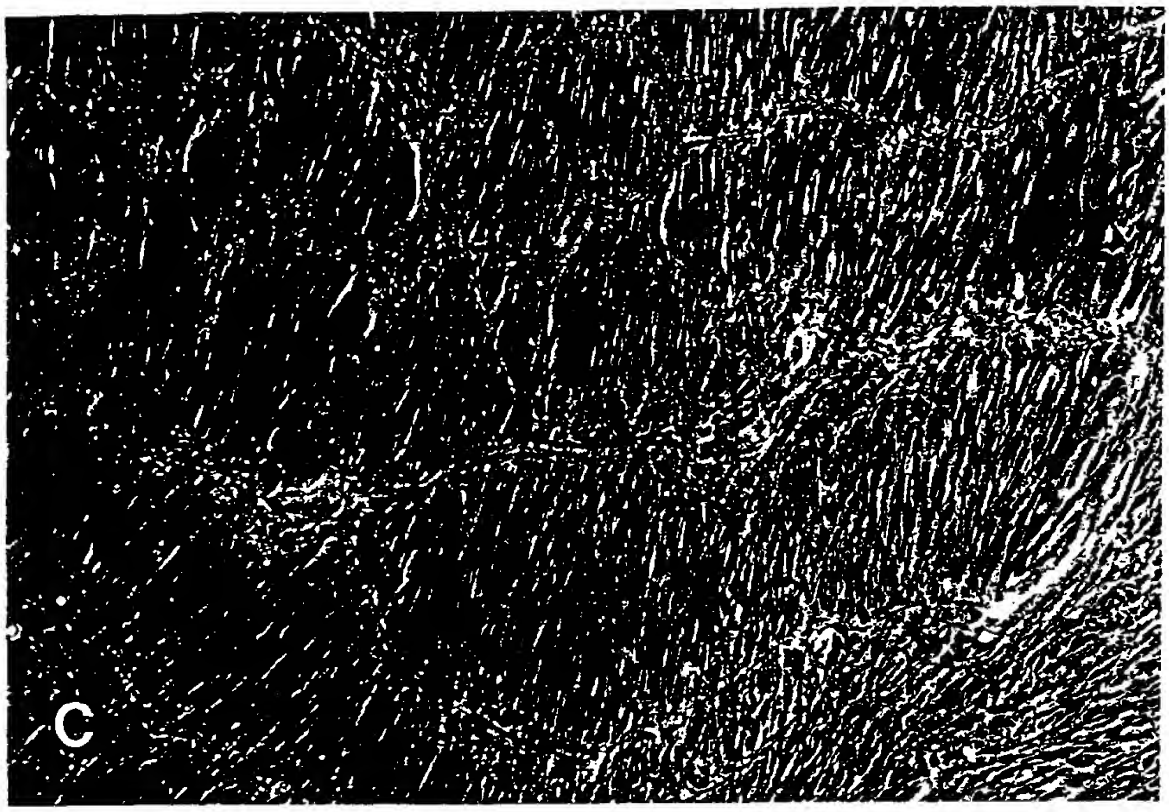


Fig. 2. A: Floating embryoid body following aggregation of rabbit ES cells. B: Pigmented embryoid body. Note the accumulation of pigment in the cytoplasm of a clone of cells proliferating on one-half of the explanted mass. ×40. (Figure 2 continued on next page.)



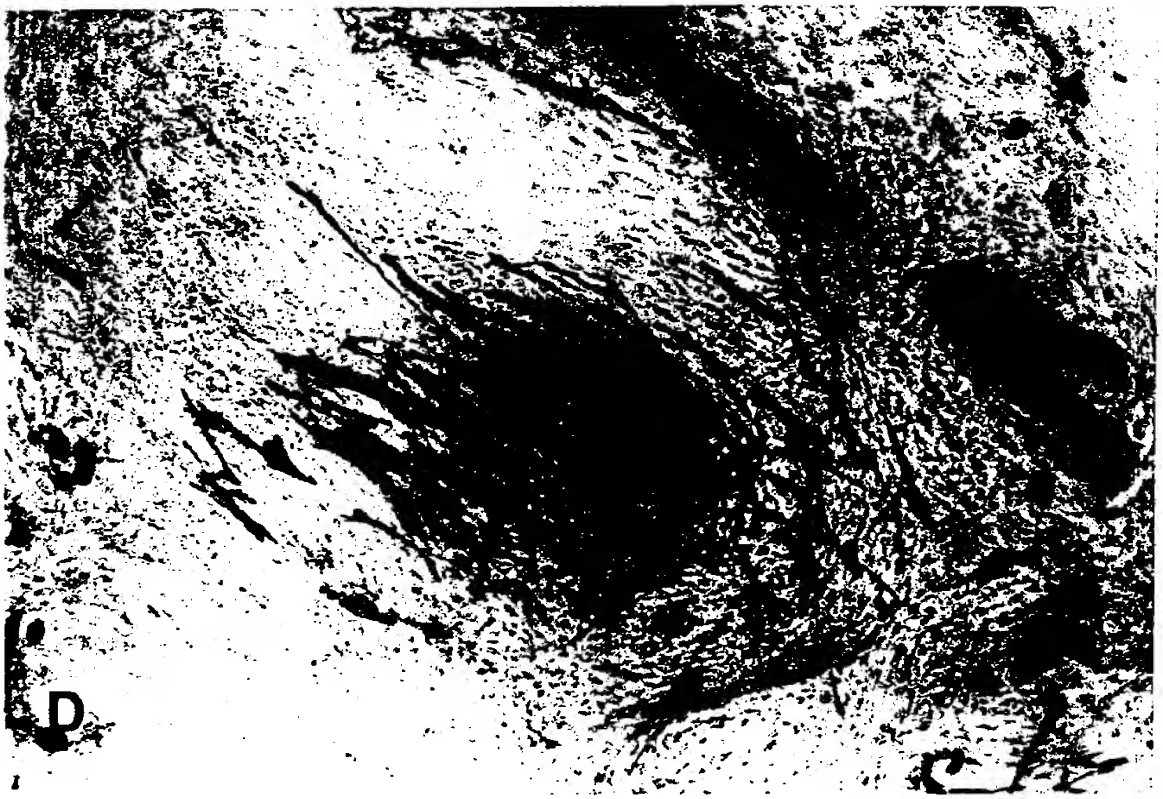


Fig. 2. C: Formation of multinucleated myotubes, which align along an axis. D: Binucleate and mononucleate cells in a culture of spontaneously contracting embryoid bodies which stain positively with anti-desmin antibodies. ×40.

Fig. 3. Electron micrographs of embryoid bodies demonstrating ciliated epithelium (A) and striated muscle (B). ×10,000.

phology and ability to form vesicles spontaneously. The second cell type is most likely derived from the inner cell mass. Both cell types are strictly feeder dependent and appear to be immortal, having been maintained in culture in an undifferentiated state for >1 year. No apparent differences have been noted in our ability to derive these cell lines from either day 4 or day 5 embryos (see Table 1). At these early developmental time points (expanded blastocysts), the inner cell mass is quite distinct, as is the trophectodermal layer, but we have not been able to discern the actual number of cells in either. These blastocysts do not normally implant into the uterine wall for another 3–4 days, and as such represent a time when rapid cell division of the inner cell mass is taking place. This is probably important for the derivation of independent cell lines (trophectoderm versus inner cell mass).

Under conditions that promote differentiation (embryoid bodies), the rabbit stem cells (early or late passage) display cell types representative of ectoderm (neural crest), mesoderm, and endoderm. These properties define their pluripotential nature, and in this respect they are similar to ES cells derived from the mouse and other species. However, it remains to be tested whether these, as well as other presumptive ES cells (hamster, pig, sheep, cow, and mink), are totipotent; that is, will the cells give rise to viable progeny when introduced into recipient blastocysts?

Several lines of evidence suggest that this goal is feasible, particularly in the rabbit. Chimeric rabbits have been obtained from injection of freshly isolated (not cultured) inner cell mass cells into recipient blastocysts (Gardner and Munro, 1974; Moustafa, 1974; Babinet and Bordenave, 1980). More recently, both freshly isolated and briefly cultured (3 days) inner cell mass cells have been reintroduced into recipient blastocysts and germline chimeric rabbits have been obtained (Yang and Foote, 1988; Giles et al., 1993; R.H. Foote, personal communication). Furthermore, the established cell lines we have described are morphologically identical to briefly cultured inner cell mass cells (Giles et al., 1993). So far, however, efforts to produce chimeric rabbits by introducing these cells into blastocysts from the NZW strain have failed; this may represent a strain barrier, and there is precedent for this in the rabbit (Moustafa, 1974). Subsequent studies will focus on introducing these into blastocysts from the albino Dutch strain (Giles et al., 1993) in order to circumvent any strain differences. In addition, we have recently generated a dominant coat color allele in the NZW strain by introducing the tyrosinase gene into one-cell embryos via DNA microinjection (founder rabbits are dark brown). Thus additional ES cell lines can be derived from these transgenic rabbit lines and introduced into recipient (albino NZW) blastocysts to circumvent completely any potential strain barriers.

At present, there are two principal methods for generating mutations in laboratory animals: the traditional method of DNA microinjection into the pronucleus of the one-cell embryo (typically producing gain-of-function mutations; for recent discussion and accompanying references, see First and Haseltine, 1991) and homologous recombination in ES cells (to produce loss-of-function mutations; see Pascoe et al., 1992). These techniques have been exploited most successfully in the mouse to dissect complex pathways of cellular differentiation and development. The consequences of mutations in genes with suspected and unknown functions can now be ascertained in an intact animal throughout its development. Indeed, the results of gene targeting have revealed functions that might never have been identified by classical biochemical or physiological methods [consider, for example, mice deficient in the low-density lipoprotein (LDL) receptor-related protein; Herz et al., 1992]. The addition of the rabbit as another species from which ES cells have been derived will provide a wealth of information about the growth properties of these cells, conditions for maintenance in an undifferentiated state, ability to direct their differentiation in vitro, and ability to introduce germline mutations. While the mouse has many obvious advantages over larger mammals for studies related to developmental biology (such as a large repertoire of existing mutations), the availability of a rabbit ES cell line with the capability to create rabbit mutations has enormous potential in the field of medicine. This has been most amply demonstrated by studies aimed at correcting hypercholesterolemia in the Watanabe rabbit (Chowdhury et al., 1991), which has a mutation in the transmembrane domain of the LDL receptor, leading to elevated levels of serum cholesterol. Indeed, gene therapy protocols have been approved for studies in humans (Wilson, 1992) as a result of successful gene therapy in this model system. At present, there are very few readily available strains of rabbits in which well-defined mutations occur.

Currently, the cloning of genes has far exceeded our understanding of the precise functions of those genes. This will become more evident in the years ahead, when sequencing of the mammalian genome is completed. Candidate genetic loci for many clinical disorders will be identified. A large animal model with which to test gain-of-function and loss-of-function mutations in these genes, and novel approaches for correction of the defects, would represent an important advance.

ACKNOWLEDGMENTS

The authors acknowledge the generous support of the Moss Heart Trust, the Perot Foundation, and an Advanced Technology Grant from the State of Texas. The technical assistance of Wille Young is greatly appreciated. The authors also recognize helpful discussions and encouragement from William Neaves, R. Sanders Williams, Michael Brown, and Joseph Goldstein.

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